

We claim:

1. A botulinum serotype A/E (BoNT/A/E) substrate, comprising:

- 5 (a) a donor fluorophore;
- (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and
- (c) a BoNT A or BoNT/E recognition sequence comprising a cleavage site,
- 10 wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

15 2. The botulinum serotype A/E substrate of claim 1 which is a BoNT/A substrate, comprising:

- (a) a donor fluorophore;
- (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said
- 20 donor fluorophore; and
- (c) a BoNT/A recognition sequence comprising a cleavage site,
- wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein,
- 25 under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

3. The substrate of claim 2, comprising at least six consecutive residues of SNAP-25, said six consecutive

30 residues comprising Gln-Arg, or a peptidomimetic thereof.

0942024-082801

4. The substrate of claim 3, comprising at least six consecutive residues of human SNAP-25, said six consecutive residues comprising Gln₁₉₇-Arg₁₉₈, or a peptidomimetic thereof.

5 5. The substrate of claim 4, comprising the amino acid sequence Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys (SEQ ID NO: 1), or a peptidomimetic thereof.

6. The substrate of claim 4, comprising residues 187 to 203 of human SNAP-25 (SEQ ID NO: 2), or a peptidomimetic thereof.

7. The botulinum serotype A/E substrate of claim 1 which is a BoNT/E substrate, comprising:

- 15 (a) a donor fluorophore;
- (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and
- (c) a BoNT/E recognition sequence comprising a cleavage site,
- 20 wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

8. The substrate of claim 7, comprising at least six consecutive residues of SNAP-25, said six consecutive residues comprising Arg-Ile, or a peptidomimetic thereof.

09042024-082801
FOB280" 42024660

9. The substrate of claim 8, comprising at least six consecutive residues of human SNAP-25, said six consecutive residues comprising Arg₁₈₀-Ile₁₈₁, or a peptidomimetic thereof.
- 5 10. The substrate of claim 9, comprising the amino acid sequence Gln-Ile-Asp-Arg-Ile-Met-Glu-Lys (SEQ ID NO: 8), or a peptidomimetic thereof.
- 10 11. The substrate of claim 9, comprising residues 156 to 186 of human SNAP-25 (SEQ ID NO: 2), or a peptidomimetic thereof.
12. The substrate of any of claims 1, 2 or 7, wherein said substrate can be cleaved with an activity of at least 1 nanomole/minute/milligram toxin.
- 15 13. The substrate of any of claims 1, 2 or 7, wherein said substrate can be cleaved with an activity of at least 20 nanomoles/minute/milligram toxin.
14. The substrate of any of claims 1, 2 or 7, wherein said substrate can be cleaved with an activity of at least 50 nanomoles/minute/milligram toxin.
- 20 15. The substrate of any of claims 1, 2 or 7, wherein said substrate can be cleaved with an activity of at least 100 nanomoles/minute/milligram toxin.
16. The substrate of any of claims 1, 2 or 7, wherein said substrate can be cleaved with an activity of at
- 25 least 150 nanomoles/minute/milligram toxin.
17. The substrate of claim 1, wherein said acceptor is an acceptor fluorophore.

T08280-42024660

18. The substrate of claim 17, wherein said acceptor fluorophore has a fluorescent lifetime of at least 1 microsecond.

19. The substrate of claim 1, wherein said acceptor is non-fluorescent.

20. The substrate of claim 1, wherein said donor fluorophore is fluorescein.

21. The substrate of claim 1, wherein said donor fluorophore is Alexa Fluor[®].

22. The substrate of claim 1, wherein said donor fluorophore is DABCYL.

23. The substrate of claim 1, wherein said donor fluorophore is BODIPY.

24. The substrate of claim 1, claim 20, or claim 21, wherein said acceptor is tetramethylrhodamine.

25. The substrate of claim 1 or claim 22, wherein said acceptor is EDANS.

26. The substrate of claim 1, claim 20 or claim 21, wherein said acceptor is QSY[®] 7.

27. The substrate of claim 1, which is a peptide or peptidomimetic having at most 100 residues.

28. The substrate of claim 27, which is a peptide or peptidomimetic having at most 50 residues.

094204.08801
T08280-42024660

29. The substrate of claim 28, which is a peptide or peptidomimetic having at most 40 residues.

30. The substrate of claim 29, which is a peptide or peptidomimetic having at most 20 residues.

5 31. The substrate of claim 1, wherein said donor fluorophore and said acceptor fluorophore are separated by at most fifteen residues.

10 32. The substrate of claim 31, wherein said donor fluorophore and said acceptor fluorophore are separated by at most ten residues.

33. The substrate of claim 32, wherein said donor fluorophore and said acceptor fluorophore are separated by at most eight residues.

15 34. The substrate of claim 33, wherein said donor fluorophore and said acceptor fluorophore are separated by at most six residues.

000042024-082801

35. A method of determining protease activity of botulinum toxin serotype A or serotype E (BoNT/A/E), comprising the steps of:

- 5 (a) treating a sample, under conditions suitable for clostridial toxin protease activity, with a BoNT/A or BoNT/E substrate comprising
 - (i) a donor fluorophore;
 - (ii) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and
 - 10 (iii) a BoNT/A or BoNT/E recognition sequence comprising a cleavage site,
- wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein, under
- 15 the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor;
 - (b) exciting said donor fluorophore; and
 - (c) determining resonance energy transfer of said
 - 20 treated substrate relative to a control substrate,

wherein a difference in resonance energy transfer of said treated substrate as compared to said control substrate is indicative of BoNT/A or BoNT/E protease activity.

25 36. The method of claim 35, wherein said botulinum toxin substrate is a BoNT/A substrate comprising a BoNT/A recognition sequence.

37. The method of claim 35, wherein said botulinum toxin substrate is a BoNT/E substrate comprising a BoNT/E

30 recognition sequence.

094204-082801
108280-42024650

38. The method of claim 35, wherein said sample is a crude cell lysate.

39. The method of claim 35, 36 or 37, wherein said sample is isolated clostridial toxin.

5 40. The method of claim 35, 36 or 37, wherein said sample is isolated clostridial toxin light chain.

41. The method of claim 35, wherein said sample is a formulated clostridial toxin product.

10 42. The method of claim 35, wherein said sample is BOTOX®.

43. The method of claim 35, step (c) comprising detecting donor fluorescence intensity of said treated substrate,

15 wherein increased donor fluorescence intensity of said treated substrate as compared to said control substrate is indicative of clostridial toxin protease activity.

20 44. The method of claim 35, step (c) comprising detecting acceptor fluorescence intensity of said treated substrate,

wherein decreased acceptor fluorescence intensity of said treated substrate as compared to said control substrate is indicative of clostridial toxin protease activity.

0094204-082801
T08280-4202450

45. The method of ~~claim 35~~, step (c) comprising detecting an acceptor emission maximum and a donor fluorophore emission maximum,

wherein a shift in emission maxima from near said
5 acceptor emission maximum to near said donor fluorophore emission maximum is indicative of clostridial toxin protease activity.

46. The method of ~~claim 35~~, step (c) comprising detecting the ratio of ~~fluorescence~~ amplitudes near an
10 acceptor emission maximum to the fluorescence amplitudes near a donor fluorophore emission maximum,

wherein a decreased ratio of said treated sample as compared to said control sample is indicative of clostridial toxin protease activity.

15 47. The method of ~~claim 35~~, step (c) comprising detecting the excited state lifetime of the donor fluorophore of said treated substrate, wherein an increased donor fluorophore excited state lifetime of said treated substrate as compared to said control
20 substrate is indicative of clostridial toxin protease activity.

48. The method of ~~claim 35~~, further comprising repeating step (c) at one or more later time intervals.

49. The method of ~~claim 35~~, wherein at least 90% of said
25 clostridial toxin substrate is cleaved.

50. The method of ~~claim 35~~, wherein at most 25% of said clostridial toxin substrate ~~is~~ cleaved.

51. The method of ~~claim 50~~, wherein at most 15% of said clostridial toxin substrate ~~is~~ cleaved.

0094204 0880
T08280 12021660

52. The method of claim 51, wherein at most 5% of said clostridial toxin-substrate is cleaved.

53. The method of claim 35, wherein the conditions suitable for clostridial toxin protease activity are
5 selected such that the assay is linear.

108280-12024660